STUDIES TO DETECT INFECTION OF LEPUS CALIFORNICUS MELANOTIS, MEARNS (BLACK-TAILED JACK RABBIT) WITH PASTEURELLA TULARENSIS, PASTEURELLA PESTIS, BRUCELLA ABORTUS AND COXIELLA BURNETII

by

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INTRODUCTION

Jack rabbits have been incriminated as possible sources of such pathogenic microorganisms as Pasteurella tularensis, P. pestis, Brucella abortus and Coxiella burnetii. Since the black-tailed jack rabbit, Lepus californicus melanotis, Mearns, (PLATE I) is numerous in western Kansas a study was undertaken in an effort to determine if this animal might be a source of infection for humans or domestic animals. Due to their concentration, jack rabbits are extensively hunted in western Kansas. This hunting serves as a source of recreation as well as an attempt to control their numbers and thus limit the crop damage done by this pest. In addition, in some areas the jack rabbit carcasses are sold for processing into food for mink farms.

Since tularemia is endemic in Kansas, jack rabbits could represent a source of infection for the hunter or the individual who processes carcasses for commercial purposes. Also, plague has been reported in western Kansas (Wayson, 1947). Since jack rabbits are susceptible to P. pestis (Vest, 1959) their relatively dense population in this area represent a potential focus for acute outbreaks of the disease to occur. Also, jack rabbits are often heavily concentrated where domestic animals feed, and the cattle could possibly become infected with Q-fever from diseased rabbits through inhalation of dust contaminated with infected secreta or excreta or from ticks that have fed on infected jack rabbits. Man could also become infected with C. burnetii in the same manner or by contact with infected cattle. Since jack rabbits are known to harbor Br. abortus their close contact with cattle could allow them to spread the disease from one area to another or to serve as a reservoir of

EXPLANATION OF PLATE I

Lacuna californiana melanella, Pearse

PLATE 1

infection. This also represents another potential hazard to man if he is to handle the jack rabbit.

In addition, a study in progress at this institution was interested in understanding the population fluctuations of the black-tailed jack rabbit in Kansas. It was felt that the presence or absence of disease within the population under study could influence this fluctuation. Consequently, an effort was made to determine whether the above diseases were present in the population.

In an attempt to ascertain the presence of the above diseases in the jack rabbit population blood and/or spleen specimens were collected from a total of 668 black-tailed jack rabbits in southwestern Kansas. These specimens were collected monthly for the duration of the two-year study.

REVIEW OF LITERATURE

P. tularensis

The etiological agent of tularemia was first described by McCoy and Chapin (1912a and 1912b) following their investigation of what McCoy (1911) called a "plague-like disease of rodents", then found among the California ground squirrels in Tulare County, California. In 1914 Wherry (1914) isolated the organism from two wild cottontail rabbits found dead in southern Indiana. This isolation was the first bacteriologic proof of a rabbit reservoir of P. tularensis. Francis (1921) later isolated the microorganism from 17 jack rabbits in Utah thus demonstrating their ability to harbor this pathogen. In Kansas, Brown et al. (1933) reported that 105 of 120 cases of tularemia from 1928 to 1933 were from skinning or handling wild rabbits. Of the 105 cases reported, seven were from contact

with jack rabbits. Since then, there have been several reports of the isolation of P. tularensis from the tissues of infected jack rabbits. Philip et al. (1935) isolated P. tularensis from the spleen and liver of two white-tailed jack rabbits during an epizootic in sheep in Montana. Parker and Smith (1955) isolated P. tularensis from a black-tailed jack rabbit tissue pool collected at Goshen, Utah in July, 1953. This pool contained spleen and liver tissue from one to five animals. The examination consisted of inoculation of the macerated tissue into guinea pigs. Using the same method of isolation, Woodbury (1955) has reported the isolation of P. tularensis from the tissues of five black-tailed jack rabbits collected in Utah between May and August, 1954. Vest (1959), in a later report from the above Utah survey, isolated P. tularensis from the bone marrow of a jack rabbit found dead June 12, 1958. The organism was recovered by inoculation of the bone marrow into deer mice. Bacon and Drake (1958) inoculated liver and spleen tissues from 421 black-tailed jack rabbits killed in Washington into white mice. They found one rabbit, killed in May, 1955, to be infected with P. tularensis. Stoenner et al. (1959) have also reported the occurrence of P. tularensis in six pools of tissues from a total of 1,459 black-tailed jack rabbits tested. Each pool consisted of lung, liver, spleen, and kidney tissue from two jack rabbits. Isolations were made by injecting tissue homogenates into guinea pigs.

Further evidence that the wild black-tailed jack rabbit can be naturally infected with P. tularensis is found in studies designed to isolate the pathogen from ectoparasites taken from specimens collected in the field. Some of the above investigators (Philip et al., 1935; Parker and Smith, 1955; Woodbury, 1955; Bacon and Drake, 1958; Vest, 1959; and Stoenner et al., 1959) also isolated P. tularensis from ticks taken from jack rabbits during their studies. Furthermore, Philip et al. (1955) were

able to isolate the pathogen from three of 14 pools of ticks removed from black-tailed jack rabbits in Nevada in 1951 and from two of 24 pools collected in 1952. Bacteriological studies on tissue specimens were not attempted in this investigation. On the other hand, Miller and Drake (1954) collected tissues from 374 black-tailed jack rabbits during a two year study in Washington and failed to isolate P. tularensis from the spleen and liver. The tissue specimens were tested by inoculation into guinea pigs or mice.

Attempts to demonstrate agglutinins against P. tularensis in the sera of jack rabbits have, in general, been unsuccessful. Miller and Drake (1954) tested the sera from an unreported percentage of 374 black-tailed jack rabbits and did not demonstrate agglutinins in any of the specimens. Also, Philip et al. (1955) failed to show agglutinins against P. tularensis in the sera from 476 black-tailed jack rabbits killed during a two-year study in Nevada. Lechleitner (1959), in a twelve-month survey of black-tailed jack rabbits in California, reported that 142 serum samples were negative for agglutinins against P. tularensis. Stoenner et al. (1959) collected sera from 773 black-tailed jack rabbits killed in Utah from 1954 to 1957. All of these samples were negative for agglutinins against P. tularensis. In addition, Vest (1959) reported that all serological tests for P. tularensis agglutinins in jack rabbits collected in Utah over a five-year period were negative. On the other hand, Bacon and Drake (1958) were able to demonstrate agglutinins against P. tularensis in the sera of two of 558 black-tailed jack rabbits killed in central Washington from 1953 to 1956. The two rabbits, collected February 13, 1955, yielded sera with a titer of 1:320. The tissues and ectoparasites of these two animals were negative for P. tularensis.

P. pestis

Although usually associated with rodents, P. pestis agglutinins have been reported in the sera taken from jack rabbits. Miller and Drake (1954) obtained plague positive antisera from five jack rabbits in the Columbia Basin in 1952 and found a single positive jack rabbit in the same area in 1953. In a continuation of the above study, Bacon and Drake (1958) found one of 35 white-tailed jack rabbits with a positive titer for P. pestis. This animal, collected in 1954, had a titer of 1:1280. However, 421 black-tailed jack rabbits collected from 1953-1956, in this same survey, were negative for agglutinins against P. pestis.

Several attempts to isolate P. pestis from the tissues of jack rabbits have been negative. Wayson (1947) reported that specimens collected from jack rabbits during a 10 year study in western United States were negative for P. pestis. Link (1950) also included the fleas from 337 jack rabbits in his 15 year survey for plague in cottontails and reported that all were negative. Also, even though Miller and Drake (1954) and Bacon and Drake (1958) found P. pestis agglutinins in seven jack rabbits during their studies, they failed to isolate P. pestis from the tissues of these jack rabbits or the other 936 tested. In each survey cited, isolations were attempted by inoculation of tissue pools into laboratory animals. Philip et al. (1955) during their study in Nevada failed to detect antigens of P. tularensis or P. pestis in the mummified carcasses of jack rabbits by the Ascoli method.

Br. abortus

Jack rabbits have also been implicated as a possible reservoir for Br. abortus. Jacotot et al. (1951) observed brucellosis in wild hares in France. Also, Philip et al. (1955) reported finding Brucella agglutinins in the sera of three black-tailed jack rabbits from one farm in Nevada. However, Lechleitner (1959) in testing 142 sera taken from jack rabbits in the Sacramento Valley of California, failed to find any sera positive for Br. abortus. Stoenner et al. (1959) also reported that 773 jack rabbit sera were tested for Brucella agglutinins and all were negative.

C. burnetii

Agglutinins against C. burnetii have been reported in jack rabbits in the United States. Luoto (1958) has tested hundreds of jack rabbit serum samples for C. burnetii agglutinins and has found several positive. Lechleitner (1959) tested 142 jack rabbits for agglutinins against C. burnetii and found five with a titer of 1:32. Stoenner et al. (1959) in testing jack rabbit sera by the complement fixation test reported that two of 773 contained antibodies against this antigen.

EXPERIMENTAL METHODS

Collection of Specimens

Field specimens were collected monthly from September 1957 to August 1959. The jack rabbits were killed in the Arkansas River Valley within a 20 mile radius of Lakin in southwestern Kansas.

The jack rabbits were taken between the hours of 3:00 p.m. and 3:00 a.m. by means of a .22 caliber rifle. Most of the hunting was from an automobile. Consequently the majority of the specimens were taken from wheat pastures, stubble fields and fallow land adjacent to county roads in the less densely populated areas near Lakin. A spotlight was used to aid in collecting after sunset.

Blood samples for the serological examinations were taken by cardiac puncture immediately after the rabbit was killed. Usually the heart continued to beat for several minutes after the rabbit was shot. Therefore, it could be readily palpated on the left side of the sternum from the level of the third to sixth rib. The blood was removed with a 10 ml syringe using a one and one-half inch, 14 - 20 gauge needle (PLATE II). The blood obtained, usually 10 ml or less, was transferred to a sterile screw-cap test tube and allowed to clot. After clotting, the blood was rimmed with a wooden applicator stick and placed in an ice chest overnight. After centrifugation the serum was removed from the test tube and returned to the laboratory in individually marked sterile vials for examination. The serum samples were stored in an ice chest for 24-48 hours while in the field and placed in the freezing compartment of a refrigerator for storage at -18° C. in the laboratory. The sera were tested for antibodies against P. tularensis, P. pestis, Br. abortus and C. burnetii.

The bacteriological examinations were made on spleens. Spleens were removed as aseptically as possible from all the jack rabbits that were not "gut shot" or in some other way showed contamination of the abdominal cavity. These tissues were collected in a field laboratory from one-to-four hours after the rabbits were shot.

In order to remove the spleen, the dead animal was placed on it's back and a lateral skin incision was made with scissors posterior to the umbilicus. The skin was then pulled forward manually, exposing the linea alba and the external abdominal muscles. A midline incision was made into the abdominal cavity from the brim of the pelvis to the xiphoid cartilage with enteron scissors that had been placed in 70 per cent ethyl alcohol. The rabbit was then rolled slightly to it's right side and a lateral incision was made just posterior to the last rib and another just anterior to the brim of the pelvis. The flap of abdominal muscles and peritoneum thus formed was folded laterally exposing the stomach and intestines on the left side of the abdominal cavity. Frequently, the apex of the spleen was exposed along the greater curvature of the stomach (PLATE II). If not, it could be easily located by moving the intestines away from the stomach and rotating the stomach anteriad. The exposed spleen was grasped with a pair of forceps and removed by cutting the gastro-splenic omentum from its medial surface and cutting the spleen just anterior to the suspensory ligament of the spleen. A second set of instruments was removed from the 70 per cent alcohol and used for manipulating the contents of the abdominal cavity and removing the spleen.

The excised portion of the spleen was placed in a sterile vial, covered with two to three ml of glycerin, and stored individually in an ice chest or refrigerator until it was examined in the laboratory. Forty-eight to 72 hours after collection the spleens were cultured for P. tularensis and P. pestis.

EXPLANATION OF PLATE II

Figure 1. Removal of blood from a jack rabbit

Figure 2. Spleen in situ along the greater curvature of the stomach

PLATE II

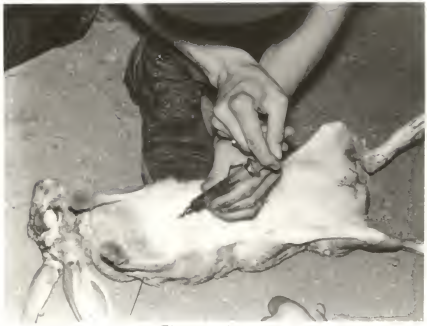

Figure 1

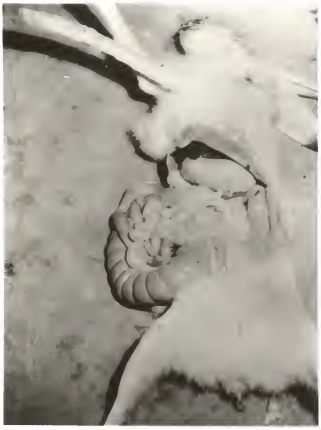

Figure 2

Serological Examinations

P. tularensis. The antigen was prepared from strain 38 of P. tularensis obtained from the laboratory of Dr. Cora M. Downs at the University of Kansas. The organism was grown on the agar surface of the modified Snyder's medium used for the isolation experiments reported in this thesis. After inoculation, the medium was incubated 72 hours at 37° C. After observing microscopically for possible contamination, the bacterial growth was washed off the agar surface with physiological saline containing 0.5 per cent formalin. The suspension of cells was centrifuged in a refrigerated centrifuge at 10° C., resuspended in formalized saline and washed a second time. The cells were then resuspended in the original volume of formalized saline, filtered through gauze to remove any clumps, and used as the stock solution of antigen. This stock solution was diluted to a concentration comparable to a McFarland standard No. 3 when the antigen was added to the diluted sera and to a McFarland standard No. 2 when the dilutions were made with antigen. Standardizations were made with a Bausch and Lomb spectrophotometer. Using a wave length of 600 μ it was found that an optical density of 23 was equal to a McFarland standard No. 3 and an optical density of 33 was equal to a McFarland standard No. 2.

The agglutination test was performed by adding 0.5 ml amounts of tularemia antigen to doubling dilutions of serum in physiological saline. The serum dilutions were prepared by adding 0.1 ml serum to 0.9 ml saline, mixing and transfering 0.5 ml to the next tube containing 0.5 ml physiological saline. A five-tube test was employed giving a serum dilution range of 1:20 to 1:320 after addition of the antigen. The first 239 sera samples were tested by this method. In July, 1958, the procedure

was changed in that 0.2 ml of serum and 0.8 ml of physiological saline were employed for the first dilution. The final serum dilutions then became 1:10 to 1:160. Starting in April, 1959 (the last 66 samples tested) the serum dilutions were made with antigen rather than physiological saline, resulting in final dilutions of 1:5 to 1:80. A positive serum control was included with each set of sera tested assuring the sensitivity of the antigen. The antigen was also combined with a negative serum in a 1:10 dilution as a means of assuring specificity of the antigen. A tube of antigen containing no serum was included so that any spontaneous agglutination could be detected. The tubes were placed in a 37° C. water bath for two hours and a preliminary reading was made. The tubes were then transferred to an ice box and incubated at 8° C. overnight. The final reading was made the following morning. Following incubation, the tubes were observed for the extent of agglutination as follows:

++++ agglutination -- complete clumping of the bacteria in the bottom of the tube with a clear supernatant fluid.

+++ agglutination -- almost complete clumping of the bacteria in the bottom of the tube with a faintly turbid supernatant fluid.

++ agglutination -- partial clumping of the bacteria in the bottom of the tube with a moderately turbid supernatant fluid.

+ agglutination -- slight clumping of the bacteria in the bottom of the tube with a turbid supernatant fluid.

- agglutination -- a smooth settling of the bacteria in the bottom of the tube with no clumping and a turbid supernatant fluid.

P. pestis. P. pestis antigen, supplied by Dr. S. F. Quan from the Communicable Disease Center, San Francisco Field Station, San Francisco 18, California, consisted of a concentrated mixture of Ireka and 14 other strains suspended in 50 per cent glycerin. A portion of this was diluted 1:60 in physiological saline each day and used as the test antigen.

A microslide agglutination test, as described by Quan (1959) was used for the detection of P. pestis agglutinins in the sera. The serum samples were inactivated at 60° C. for 30 minutes and then diluted 1:10, 1:20, 1:40, and 1:80 in physiological saline. Five-hundredths ml of each dilution was transferred with a tuberculin syringe and a 26 gauge, one-quarter inch needle to a syphilis flocculation slide containing 0.05 ml of the diluted P. pestis suspension. Rabbit anti-plague globulin, furnished by Dr. Quan, was employed to assure the sensitivity of the antigen. The results were read under 100 X magnification after the mixtures on the slide had been gently rotated at room temperature for 30 minutes.

Br. abortus. The Br. abortus antigen, kindly supplied by Mr. Edward Roznowski, Jr., was prepared from a culture of Br. abortus obtained from the collection of Dr. K. J. McMahon of the Department of Bacteriology, Kansas State University. The Br. abortus culture was grown on potato agar slants. This medium was prepared by slicing 250 grams of raw potatoes in 1000 ml of water, holding the mixture overnight at approximately 60° C., and then filtering it through filter paper. The filtrate was made up to 1000 ml with distilled water and the following ingredients added:

Sodium Chloride, U.S.P. . . . . 5 grs
Bacto-peptone . . . . . .10 grs
Beef extract. . . . . 5 grs
Dextrose, U.S.P.. . . . . .10 grs
Agar, U.S.P.. . . . . .30 grs

The medium was heated in a steamer to dissolve the agar, and 20 ml of glycerin, U.S.P. were added. Adjustment of the medium to pH 7.5 resulted, after autoclaving, in a final pH of 6.8. The medium was filtered through a pad composed of two thin layers of absorbent cotton and dispensed into tubes, stoppered and autoclaved for 30 minutes at 120° C. The growth from the agar slants was suspended in five ml of physiological saline and used as the seed culture for the antigen production. Blake bottles containing 100 ml of this potato agar were inoculated with the Br. abortus and incubated 72 hours at 37° C. After observing microscopically for contamination, the bacterial growth was washed off the agar surface with 10 ml of phenolized saline (0.25 per cent phenol in physiological saline) and filtered through gauze to remove any clumps of bacteria or culture medium. The filtrate was steamed 10 minutes and stored in a refrigerator at approximately 8° C. Standardized Br. abortus antigen was prepared from this filtrate.

The antigen was standardized against a Brucella tube antigen obtained from Dr. Louis H. Smith, Veterinarian in charge of the United States Department of Agriculture, Animal Research Service, Topeka, Kansas. The standardization was performed in a Bausch and Lomb spectrophotometer at a wave length of 600 $\mu\mu$. In addition, the standardized antigen was compared to the United States Department of Agriculture standard in ability to be agglutinated by a positive serum sample.

This serum sample demonstrated the same tube agglutination titer with each antigen, indicating that the sensitivity of the prepared antigen compared favorably with the standard antigen.

A three-tube agglutination test was used for the detection of Brucella agglutinins. In performing the test, 0.2 ml of serum was diluted with 1.8 ml of antigen resulting in final dilutions of 1:10, 1:20 and 1:40. The antigen was also combined with a negative serum in a 1:10 dilution as a means of assuring specificity of the antigen. A tube of antigen containing no serum was included so that any spontaneous agglutination could be detected. The tubes were incubated at 37° C. for 40-48 hours and read the same as the P. tularensis agglutination tests described above.

C. burnetii. The antigen used in the capillary-tube agglutination test for C. burnetii was supplied by Dr. L. Luoto, from the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, Rocky Mountain Laboratory, Hamilton, Montana. California strain No. 16 (Luoto, 1953) of C. burnetii was used in the preparation of this antigen as described by Luoto (1955). The organism was inoculated into the yolk sacs of fertile eggs previously incubated for six days at 100-101° F. On the sixth and seventh day postinoculum the eggs were candled and the yolk sacs of viable embryos harvested. These yolk sacs were diluted to 60 per cent of their original weight in sterile distilled water and homogenized for two to three minutes in a Waring Blendor. This suspension could be used immediately or stored indefinitely in a dry-ice chest. To prepare the antigen from this stock suspension 0.5 ml of formalin was added to every 100 grams of original yolk-sac material and the mixture allowed to stand

overnight at room temperature. A 30 per cent suspension then was prepared by dilution of the formalin treated suspension with an equal volume of physiological saline containing 1.5 per cent phenol. After standing at room temperature 24-72 hours, the phenolized suspension was diluted to a final concentration of 10 per cent yolk-sac. This was then extracted with one and one-half volumes of diethyl ether at 4° C. for four to six hours. The aqueous phase was removed and the extraneous yolk-sac material was separated from the rickettsiae in the suspension by low speed centrifugation for 10 minutes. The supernatant fluid was drawn off and subjected to high speed centrifugation for one hour. This differential centrifugation was repeated and the sediment was resuspended to its original volume in formalized saline. A modified Harris stain containing an increased amount of hematoxylin was utilized for staining the antigen. One part of stain was added to 10 parts of antigen, and the mixture was allowed to stand 48 hours at 60° C. or 72 hours at 37° C. The stained antigen was washed by differential centrifugation and resuspended in distilled water containing 0.2 per cent formalin and buffered at pH 7.0. This stock antigen was standarized to 35-45 per cent of light transmission using a Coleman Jr. spectrophotometer, Model 6A, employing a wave length of 6,500 Å. The standardized antigen is stable and ready to use in the capillary agglutination tests.

In performing the test, a capillary tube (0.4 mm by 900 mm) was filled approximately one-third full by placing it into an aliquot of the stained antigen. The capillary tube was then placed into a sample of undiluted serum and allowed to fill automatically by capillary action. The tube was inverted (antigen at the bottom) and placed in a vertical position in clay. After four hours incubation at room temperature the

tests were read under a direct light source. A positive reaction was indicated by blue-black, macroscopic agglomerates of C. burnetii produced in the presence of specific antibody. In a negative reaction no such macroscopic particles were observed. Positive bovine serum and positive bovine milk were used in each set of sera tested assuring the sensitivity of the antigen. A tube of antigen containing no serum was included so that any spontaneous agglutination could be detected, and a negative serum was run to assure specificity of the antigen.

Bacteriological Examinations

P. tularensis. A modified solid Snyder's medium was used for the detection of P. tularensis in the spleens. The medium was prepared from the following concentrate which was stored at 5.0° C. until needed:

Difco Proteose Peptone	100 grs
Basamin-Busch Yeast Autolysate (Bis #7120) Anheuser-Busch, Inc.	100 grs
Bacto-dextrose	250 grs
NaCl, U.S.P.	100 grs
Distilled Water	q.s. 1000 ml

In the preparation of 100 ml of the medium 0.1 gram of cysteine hydrochloride was added to a 1:10 dilution of the concentrate and the pH adjusted to 7.0. After the addition of 1.5 per cent agar the medium was cooled to 50° C. and 5 per cent citrated human blood was added (out dated blood bank blood was obtained for this purpose). During the second year a new, blood free, medium (Won, 1958) was also employed for the detection of P. tularensis in the splenic tissue. The composition of 100 ml of this medium is as follows:

Spermidine Phosphate (Nutritional Biochemical Co)	1.0 mg
N-Acetyl-glucosamine (Mann)	100.0 mgs
D-Histidine HCl	0.5 gr
DL-Glutamic Acid	100.0 mgs
NaCl	0.5 gr
Yeast Extract (Difco)	0.5 gr
Peptone (Difco)	1.0 gr
Orotic Acid (Nutritional Biochemical Co)	10.0 mgs
Agar	1.5 grs
Glucose (50 per cent)	2.0 ml
Cysteine HCl (20 per cent) (Eastman)	0.5 ml

The ingredients were compounded in the order given. After the addition of orotic acid, the incomplete medium was adjusted to pH 7.2 with 10 N NaOH. Agar then was added and the medium was autoclaved 15 minutes at 120° C. After cooling, autoclaved glucose and filtered cysteine were added asceptically and the pH readjusted to 7.3 when necessary.

Agar plates of these media were streaked with the cut surface of the spleens after excess glycerin was removed by washing in sterile physiological saline. In addition, beginning April, 1958, ground splenic material was used as an inoculum. A small portion of the spleen was placed into a 16 by 150 mm glass tissue homogenizer with three to four ml of physiological saline and reduced to a fine particulate suspension. This homogenate was poured over the surface of the agar plates. The plates were incubated at 37° C. for five days and examined daily for evidence of growth.

P. pestis. Blood agar was used for the detection of P. pestis in the spleens. Difco tryptose agar with five per cent citrated human blood was the medium employed (out dated blood bank blood was obtained for this purpose). One liter of this medium has the following composition:

Bacto-tryptose	20.0 grs
Bacto-dextrose	1.0 gr
NaCl	5.0 grs
Bacto-agar	15.0 grs
Thiamine Hydrochloride	.0.005 gr

Originally, the plates were held at 37° C., but the last 375 samples were incubated at 25-30° C., as this is considered a more optimum temperature for the growth of P. pestis. The plates were incubated for five to seven days and examined daily for evidence of growth.

EXPERIMENTAL RESULTS AND DISCUSSION

P. tularensis

A total of 496 serum samples were tested over the two years for agglutinins against P. tularensis (Table 1). All samples were negative. Negative results have also been reported by other workers (Miller and Drake, 1954; Philip et al., 1955; Lechleitner, 1959; Stoenner et al., 1959; and Vest, 1959). Bacon and Drake (1958), in a survey of eastern and central Washington, found only two positive sera of 558 tested. Vest (1959) has interpreted the lack of positive agglutinating sera during his five-year study as an indication of the lack of survivors among naturally infected animals. Jack rabbits and other mammals within the Order Lagomorpha show considerable variation in their sensitivity to

virulent strains of P. tularensis. It is generally believed that cottontails, like rodents, succumb when they are infected with P. tularensis (Bell and Chalgren, 1943); consequently, they do not generally possess antibodies in nature. However, McKeever et al., (1958) reported that one cottontail from a group of 188 collected in southwestern Georgia and northwestern Florida from 1955 to 1957 had a titer of 1:320 against P. tularensis. Several investigations have been conducted in an effort to determine the ability of the jack rabbit to develop agglutinins when exposed to virulent strains of P. tularensis. Philip et al. (1955) inoculated four black-tailed jack rabbits from Kansas with 1,000,000 organisms each and reported that only one survived long enough to produce antibodies. This study also included two jack rabbits that received 100 organisms each and neither became ill nor developed detectable antibodies after 34 days. Stagg et al. (1956) inoculated one black-tailed jack rabbit from Utah with an unknown number of P. tularensis organisms by an aerosol exposure. The animal died on the fourth day without developing antibodies. Vest (1957) reported that the black-tailed jack rabbit L. californicus texianus, Mearns was less susceptible than rodents or cottontails to P. tularensis. All animals that did not survive were dead by the sixth day. Later, (Vest, 1959) L. californicus deserticola, Mearns were inoculated subcutaneously with P. tularensis and were found to be less resistant than the closely related subspecies, L. californicus texianus, Mearns. On the other hand, snowshoe rabbits, also of the genus Lepus, do survive attacks of tularemia (Green, 1939) and commonly develop agglutinins against the microorganism (Green, 1938). Consequently, Vest (1959) has concluded that results relating to local races of rodents and rabbits may be appreciably different from those obtained in subspecies of other areas.

Table 1. Summary of specimens tested

Date specimen collected	Number of Tests Performed: Spleens cultured	Serological Tests: Tularemia	Plague	Brucellosis	Q-fever
Sept. 1957	16	24	18	21	22
Oct. "	21	31	16	29	29
Nov. "	14	19	16	15	16
Dec. "	10	13	10	10	13
Jan. 1958	23	24	16	19	23
Feb. "	22	20	18	18	20
Mar. "	13	21	20	21	21
Apr. "	29	33	31	33	33
May "	24	27	26	26	27
June "	34	25	19	24	20
July "	29	19	19	18	18
Aug. "	25	19	19	19	19
1st Yr. Subtotal	260	275	228	253	261
Sept. 1958	25	20	19	19	21
Oct. "	19	27	25	27	27
Nov. "	29	25	22	25	24
Dec. "	19	24	23	22	24
Jan. 1959	16	20	19	20	20
Feb. "	23	21	21	21	21
Mar. "	22	17	17	17	17
Apr. "	15	16	16	16	16
May "	14	17	17	17	17
June "	12	9	9	8	9
July "	16	15	15	14	15
Aug. "	8	10	9	9	10
2nd Yr. Subtotal	218	221	212	215	221
Total	478	496	440	468	482

Four hundred seventy-eight spleens were cultured in an attempt to isolate P. tularensis. All spleens were grossly negative of any macroscopic pathology and were negative upon culture. Although animal inoculation was not used for the isolation of P. tularensis, it is felt that the methods employed were sufficient to isolate organisms that might have been present in the jack rabbits tested. This assumption is based on the fact that jack rabbits (Francis, 1921), like cottontails (Francis, 1937), show innumerable small white lesions on the liver and spleen by

the third or fourth day of illness. Consequently, it is felt that had the jack rabbits been infected for any length of time, some pathology would have been noticed on the spleens. Also, culture techniques frequently have been used for the direct isolation of P. tularensis from tissues and blood (Shaw and Hunnicutt, 1931; Gudger, 1933; Foshay and Mayer, 1936; Ransmeier and Schaub, 1941; Nakomura, 1950; and McKeever et al., 1958). In addition, Larson (1954) has reported on the relative value of a liquid medium and blood cysteine glucose agar (BCGA) as compared to mouse inoculations for the titration of P. tularensis. He found that the end point for spleen suspension and heart blood cultures was either the same for each method or only one log less when culturing on BCGA as compared to mouse inoculations.

In this laboratory an experiment was designed to compare the relative efficiency of the two media used with BCGA agar and also to test the effect of refrigerator storage in glycerin on the recovery of P. tularensis from infected mouse spleens. The results, shown in Table 2, indicate that both Snyder's medium and the new blood free medium are comparable to the BCGA medium and that 48 hours storage in the refrigerator did not adversely affect the recovery of the organism.

Table 2. Efficiency of media used and the effect of refrigerator storage in glycerin on the recovery of P. tularensis from infected mouse spleens.

Storage time in glycerin in days	Preparation of spleens: Streaked Material: Sn*	Sp	BCGA	Ground Material: Sn	Sp	BCGA	Control: BA
0	4/4**	4/4	4/4	3/3	3/3	3/3	0/3
2	4/4	4/4	3/4	4/4	4/4	4/4	0/2
3	5/7		4/7	7/7	5/7	7/7	0/7
6	0/4		0/4	4/5		4/5	0/5
11				5/6	1/2	3/4	0/6

Table 2. Concluded.

* Sn Modified Snyder's Medium
Sp Blood Free Spermidine Medium
BCGA Blood Cysteine Glucose Agar
BA Nutrient Blood Agar

** 4/4 4 positive isolation /4 isolations attempted

P. pestis

A total of 440 serum samples were tested by the microslide agglutination test (Table 1). All samples tested were negative. The 478 spleens that were cultured for P. tularensis were also cultured for P. pestis. These spleen specimens were negative for P. pestis. Originally the plates were cultured at 37° C. Miller and Drake (1954), Bacon and Drake (1958), and Bacon et al. (1958) have used this temperature for the isolation of P. pestis in all their surveys in central Washington. However, others have reported that the nutritional requirements of this pathogen are less exacting at temperatures below 30° C. (Hills and Spurr, 1952; Higuchi and Carlin, 1958; and Brownlow and Wessman, 1960). Also Wessman et al. (1958) have reported that five virulent strains of P.pestis cultivated at 37° C. in a chemically defined medium containing glucose as the energy source, underwent a reduction in viable count due to some toxic principle. Consequently, since May, 1958 the isolation procedure was changed in that the culture plates were incubated at 25-30° C.

Quan (1958) compared bacteriological culture techniques with animal inoculations for the detection of P. pestis in wild rodent fleas. He cultured 1,297 fleas individually on blood agar plates at 28° C. and inoculated the same fleas as 319 pools into animals. The sensitivity of both methods was excellent. The efficiency was very good for bacteriolog-

ical culture and excellent for animal inoculation. However, the efficiency of the bacteriological culture technique did not drop when the number of organisms per flea was small, as did the efficiency of animal inoculation. In addition, the culture method allows the processing of a large number of specimens individually. Piras (1913) was able to isolate P. pestis directly onto culture media from the spleen and other tissues of naturally infected rats held at 20-25° C. through the fifth day after death. Also, Girard (1958) used both mouse inoculation and culture techniques for counting the number of P. pestis organisms in the spleen of mice that died from plague. He found that when the number of organisms was low he could isolate from mouse spleens onto culture media with more accuracy than when he isolated by mouse inoculation. In addition, Miller and Drake (1956) have shown that there is no significant loss of plague organisms from animals collected in the field and held in the refrigerator or deep freeze four days. Thus, it is felt that P. pestis would have been isolated if any of these jack rabbits had been infected with the organism.

Br. abortus

Four hundred sixty-eight serum samples were tested for antibodies against Br. abortus (Table 1), using the standard tube agglutination test. Negative results were obtained. Since the antigen used was standardized against an antigen supplied by the United States Department of Agriculture and the agglutination test was performed according to the standard methods suggested by the United States Public Health Service, it is felt that Br. abortus agglutinins would have been detected had they been present.

C. burnetii

Four hundred eighty-two serum samples were tested for antibodies against C. burnetii and all were negative. Perhaps this is not surprising since Stoenner et al. (1959) found only two of 773 jack rabbits with a titer against C. burnetii. Lechleitner (1959) did find, however, a positive titer in five of 142 jack rabbit sera tested.

The capillary-tube agglutination test used for these studies has been compared with the complement fixation test, standard rickettsial agglutination test, and the microslide agglutination test by Welsh et al. (1959). They used 1,017 serum samples from 113 sheep that were naturally exposed to C. burnetii. According to their results, the capillary-tube agglutination test, the complement fixation test, and the standard rickettsial agglutination test were comparable in respect to sensitivity and in ability to detect the appearance of antibody and to follow its persistence. The microslide agglutination test was superior to the other three tests except where the sera were used at a minimum dilution. Since tests were performed with undiluted sera it is felt that agglutinins would have been detected had any been present.

Statistical Analyses

The work reported in this thesis is summarized in Table 1. A total of 668 jack rabbits were included in this study. Upon analysis of the data with the 95 per cent confidence interval for binomial distribution, from 0 to less than 1 per cent of the population would be expected to be infected with P. tularensis or P. pestis or to have been exposed to P. tularensis, P. pestis, Br. abortus or C. burnetii and survived to

produce antibodies. The sera and spleens were collected from rabbits which represented a mixture of adults (60.5 per cent) and juveniles (39.5 per cent) (Table 3) shot randomly from the population in Kearny County, Kansas. Although 668 jack rabbits were shot, only 306 or 45.8 per cent, were subjected to both serological and bacteriological examination. One hundred seventy-two, or 25.7 per cent of the remaining rabbits tested, had only the bacteriological examination and 190, or 28.5 per cent, had only the serological examination.

Table 3. Tests performed each month by age group.

Month collected	Age of rabbit	Laboratory Tests Performed on Individual Rabbits: Bacteriology and Serology	Bacteriology only	Serology only
Sept. 1957	Juveniles	13	-	6
	Adults	3	-	2
Oct. 1957	Juveniles	18	-	8
	Adults	3	-	2
Nov. 1957	Juveniles	11	-	4
	Adults	3	-	1
Dec. 1957	Juveniles	5	-	2
	Adults	5	-	1
Jan. 1958	Juveniles	5	-	2
	Adults	12	5	5
Feb. 1958	Juveniles	-	-	-
	Adults	12	10	8
Mar. 1958	Juveniles	1	-	-
	Adults	10	2	10
Apr. 1958	Juveniles	-	-	-
	Adults	24	5	9
May 1958	Juveniles	-	2	1
	Adults	18	4	8

Table 3. Cont.

Month collected	Age of rabbit	Laboratory Tests Performed on Individual Rabbits: Bacteriology and Serology	Bacteriology only	Serology only
June 1958	Juveniles	9	8	4
	Adults	7	10	5
July 1958	Juveniles	6	14	6
	Adults	2	7	5
Aug. 1958	Juveniles	5	12	7
	Adults	5	3	2
SUBTOTAL 1st year	Juveniles	73	37	40
	Adults	104	46	58
Sept. 1958	Juveniles	10	5	5
	Adults	4	6	1
Oct. 1958	Juveniles	10	6	13
	Adults	3	-	1
Nov. 1958	Juveniles	14	7	4
	Adults	4	4	3
Dec. 1958	Juveniles	8	2	4
	Adults	8	1	4
Jan. 1959	Juveniles	-	-	-
	Adults	10	6	10
Feb. 1959	Juveniles	-	-	-
	Adults	15	8	6
Mar. 1959	Juveniles	-	-	-
	Adults	11	11	6
Apr. 1959	Juveniles	-	1	-
	Adults	7	7	9
May 1959	Juveniles	-	1	-
	Adults	7	6	10
June 1959	Juveniles	-	1	-
	Adults	7	4	2
July 1959	Juveniles	4	10	2
	Adults	2	-	7
Aug. 1959	Juveniles	4	2	1
	Adults	1	1	4

Table 3. Concl.

Month collected	Age of rabbit	Laboratory Test Performed on Individual Rabbits: Bacteriology and Serology	Bacteriology only	Serology only
SUBTOTAL 2nd year	Juveniles	50	35	29
	Adults	79	54	63
TOTAL	Juveniles	123	72	69
	Adults	183	100	121
	All rabbits	306	172	190

SUMMARY

During a two-year period (Sept. 1957-Aug. 1959) Kansas black-tailed jack rabbits were sacrificed periodically in the field, and specimens collected from them were examined in the laboratory for evidence of infectious diseases. Bacteriological examinations on spleen specimens were found to be negative for tularemia and plague. Serological tests on serum samples were negative for tularemia, plague, brucellosis, and Q-fever. Since the specimens were collected from rabbits which represented a mixture of adults and juveniles shot randomly from the population in Kearny County, Kansas, it is believed that the above diseases did not exist in this population during the two-year study.

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STUDIES TO DETECT INFECTION OF LEPUS CALIFORNICUS MELANOTIS, MEARNS (BLACK-TAILED JACK RABBIT) WITH PASTEURELLA TULARENSIS, PASTEURELLA PESTIS, BRUCELLA ABORTUS AND COXIELLA BURNETII

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Jack rabbits have been incriminated as possible sources of such pathogenic microorganisms as Pasteurella tularensis, P. pestis, Brucella abortus, and Coxiella burnetii. Since the black-tailed jack rabbit, Lepus californicus melanotis, Mearns, is numerous in western Kansas, a study was undertaken in an effort to determine if this animal might be a source of infection for humans or domestic animals. In an attempt to ascertain the presence of the above diseases in the jack rabbit population blood and/or spleen specimens were collected from a total of 668 black-tailed jack rabbits in southwestern Kansas. These specimens were collected monthly for the duration of the two-year study.

Spleen specimens were cultured on suitable media for the detection of P. tularensis and P. pestis. Standard serological procedures were utilized for the detection of antibodies against P. tularensis, P. pestis, Br. abortus, and C. burnetii. Although 668 jack rabbits were shot, only 306, or 45.8 per cent were subjected to both serological and bacteriological examination. One hundred seventy-two of the remaining rabbits tested, or 25.7 per cent, had only the bacteriological examination and 190, or 28.5 per cent, had only the serological examination. The sera and spleens were collected from a mixture of adults (60.5 per cent) and juveniles (39.5 per cent) shot randomly from the population in Kearny County, Kansas.

Bacteriological examinations on spleen specimens were found to be negative for tularemia and plague. Serological tests on serum samples were negative for tularemia, plague, brucellosis, and Q-fever. Consequently, it is believed that the above diseases did not exist in this population during the two-year study.